Positive effect of combining high pressure and biopreservation treatments on lowering the bacterial growth during storage of diced cooked ham with reduced nitrite salt

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Abstract

We investigated the combined effects of biopreservation and high pressure treatment on bacterial communities of diced cooked ham prepared with diminished nitrite salt. First, bacterial communities of four commercial brands of dice cooked ham from local supermarkets, sampled near the use-by-date, were characterised. The four ham microbiota showed a relative low diversity but harboured quite dissimilar communities. Two ham samples were dominated by different Proteobacteria (*Pseudomonas*, *Serratia* for one, *Psychrobacter* and *Vibrio* for the other one) while the two others were dominated by Firmicutes (*Latilactobacillus* and *Leuconostoc*). Second, sterile diced cooked ham, prepared with reduced level of nitrite was inoculated with the two Proteobacteria-rich microbiota collected from the aforementioned commercial samples together with a *Lactococcus lactis* protective strain. Dices were then treated at 500 MPa for 5 minutes and bacterial dynamics was monitored during storage at 8°C. Applied alone, none of the treatments stabilized durably the growth of hams microbiota. Nevertheless, the combination of biopreservation and high pressure treatment was efficient to reduce the growth of Proteobacteria spoilage species. However, this effect was dependent on the nature of the initial microbiota, showing that use of biopreservation and high pressure treatment as an alternative to nitrite reduction for ensuring cooked ham microbial safety merits attention but still requires improvement.

1. Introduction

Nitrite salts are used since ancient times as curing agents for the production of cured meat products. Nitrite (and eventually nitrate) salts are commonly added in the brine for the manufacturing of cooked ham. Their role is important for the typical pinky/reddish color development of cured meats (Alahakoon et al., 2015). Nitrite salts also participate to the hurdle technology for ensuring microbial safety as bactericidal and bacteriostatic agents against several pathogenic bacteria occurring in meat products, in particular *Clostridium botulinum* (Majou and Christieans, 2018). However, nitrite can potentially lead to carcinogenic nitrosamine production in these products, which rises the concern of

46 a potential public health risk (Sindelar and Milkowski, 2012). In European Union (EU) all additives
47 authorized before the 20th of January 2009 have to be re-evaluated by 2020. In that context, the
48 European Food Safety Authority (EFSA) re-assessed the safety of nitrite and nitrate salts in 2017. The
49 current acceptable daily intakes for nitrite and nitrate was assessed and experts stated that
50 exposure to nitrites used as food additives was within safe levels for most of the population, except
51 for children (EFSA, 2017). Nevertheless, although experts evaluated the need for further scientific
52 information, a positive link could be established between dietary intake of nitrite (or of processed
53 meat containing both nitrite and nitrate) with some cancers (Mortensen et al., 2017a, 2017b).
54 Therefore, reducing nitrite levels in processed meats appears necessary in order to limit this sanitary
55 issue. Several hurdle technologies can be proposed in order to compensate a higher risk of microbial
56 safety in cooked ham with reduced nitrite salts. Among those, High Pressure Processing (HPP) has
57 been proposed in context of food additive reduction (Pottier et al., 2017) and was shown to
58 efficiently reduce bacterial contaminants in cooked ham (Han et al., 2011; Pietrasik et al., 2016;
59 Pingen et al., 2016). As well, biopreservation, a method using protective cultures, often lactic acid
60 bacteria (LAB) or their metabolites, was described more than 20 years ago for fighting undesired
61 bacterial contaminants (Stiles, 1996). Addition of such LAB protective cultures to cooked meat
62 products, and in particular cooked ham was indeed reported (Vermeiren et al., 2006, 2004). HPP
63 combined to biopreservation has also been studied (Oliveira et al., 2015). HPP treatment of cooked
64 ham treated with bacteriocins has been shown to reduce the population of several bacterial
65 pathogens or to prolong cold storage (Jofré et al., 2008a, 2008b; Liu et al., 2012; Marcos et al., 2008;
66 Teixeira et al., 2018). In the present study, we investigated the combined effect of HPP and
67 biopreservation on the dynamics of bacterial communities of cooked ham with reduced level of
68 nitrite salts. We had previously selected a *Lactococcus lactis* strain able to recover after HPP
69 treatment and producing the bacteriocin nisin (Ramaroson et al., 2018). Here, we first determined
70 and collected bacterial communities present on commercial diced cooked ham. Then the dynamics of
71 these bacterial communities was monitored, following their inoculation in sterile diced cooked ham

with reduced nitrite level, in the presence of the bioprotective strain, and after the application of the HPP treatment.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

Lactococcus lactis CH-HP15 (Ramaroson et al., 2018) was first cultivated on M17 agar plates (Biokar diagnostic, Beauvais, France) at 30°C for 72 h. One colony was inoculated for preculture in Medium Modelling Ham (MMH) (Ramaroson et al., 2018, Modugno et al., 2019) at 30°C for 24 h, and then cultured in MMH at 30°C under agitation at 65 rpm for 13h until early stationary phase. Bacterial enumerations were performed by plating serial dilutions of bacterial cultures, microbiota or ham stomached samples. The total viable counts were determined on Plate Count Agar (PCA) (Biokar diagnostic, Beauvais, France). Psychrophilic and mesophilic counts were determined after incubation for 4 days at 15°C or 24 h at 30°C, respectively. LAB were enumerated on Man Ragosa and Sharpe (MRS) agar medium pH 5.2 (AES, France) containing bromocresol green (25 $mg \cdot l^{-1}$) as previously described after 4 days incubation at 25 °C (Najjari et al., 2008). In ham samples inoculated with *L. lactis* CH-HP15, M17 agar plates were used for enumeration after 48 h incubation at 30°C. For anaerobic conditions, plates were incubated in jars with anaerocult sachets (Anaerocult A, Merck, Germany).

2.2. Ham sampling and microbiota recovery

Diced cooked ham (except for one sample consisting of sliced cooked ham) were purchased in 2015 and 2016 from local supermarkets in Nantes, France, transported at cold temperature to the laboratory and reconditioned immediately. These samples, sold as ready-to-eat food were conditioned in packs ranging from 120 to 200 g under modified atmosphere or without any indication of any gas mixture used in the packs. At arrival in the laboratory (day 1), bags were opened and dices were immediately reconditioned as 25 g aliquots under air or vacuum packaging and incubated at 4°C. Total counts and LAB counts were enumerated at day 1, 7, 14, 21, and 28. When PCA mesophilic

97 counts reached about 7 log_{10} CFU per gram of ham, bacterial communities were collected by mixing
98 25 g diced cooked ham in 75 ml peptone salt (AES, France) for 2 min in a stomacher (Masticator, IUL
99 Instruments, England). The homogenate was filtered through the bag filter and centrifuged through a
100 filter from Nucleospin Plant II Midi kit (Macherey Nagel, EURL, France) at 8000 × g during 10 min at
101 room temperature. The bacterial pellet was resuspended in 30 ml peptone salt and aliquoted as 1 ml
102 with glycerol 15% and stored at -80°C. In total, eight microbiota were recovered from ham dices
103 either conditioned under air or vacuum.

104 **2.3. Challenge tests**

105 Low-contaminated diced cooked ham containing 18 g/kg sodium chloride and reduced level of nitrite
106 (25 mg/kg; recommended max. level is 120 mg/kg) were used for challenge tests. Those were
107 produced as previously described (Ramaroson et al., 2018). Briefly, pork ham muscles were defatted,
108 trimmed and minced using a 20 mm grid. One kilogram of this grinding was mixed under vacuum
109 with 100 g of brine containing water 74.1 g, nitrite salt 4.6 g (0.6%), sodium chloride salt 15.2 g,
110 sodium ascorbate 0.6 g, and dextrose 5.5 g. The mix was melded and vacuum-packed. Hams (2.5 kg)
111 were cooked in a 100% humidity atmosphere (90 min at 55 °C, 60 min at 60 °C and 235 min at 67 °C),
112 cooled at ambient temperature (18 °C) for 20 min and then stored at 3 °C. Ham cubes of 1 cm × 1 cm
113 were prepared aseptically, aliquoted in 100 g portions, and stored vacuum-packed at −20 °C until
114 use. Prior to microbiota inoculation, cooked ham dices were defrosted at 4 °C for 24 h and first
115 inoculated with microbiota. Two different microbiota were inoculated in low-contaminated diced
116 cooked ham. Each challenge test was performed twice (two independent experiments). Microbiota
117 previously collected were defrosted rapidly at room temperature, diluted to a final concentration of
118 6 log CFU.mL^{-1}, inoculated at 4 log CFU g^{-1} in diced cooked ham (1% v/w inoculation rate), stored
119 overnight at 4°C for allowing bacterial recovery, and then inoculated with a fresh culture of *L. lactis*
120 CH-HP15. *L. lactis* culture in early stationary phase was centrifuged. The bacterial pellet was rinsed in
121 a sterile solution of NaCl 0.9% and resuspended at 9.3 log CFU g^{-1} and 0.5 ml were inoculated in 100

122 g dice cooked ham for an initial concentration of 7 log CFU g^{-1}. Diced cooked ham inoculated with
123 microbiota but without *L. lactis* were also used as controls.

124 **2.4. HP treatments**

125 Cooked ham dices were aliquoted as 20 g portions, vacuum-packed and High-Pressure treated at
126 500 MPa for 5 min. Samples were treated using high pressure in a 3 L vertical high pressure pilot unit
127 (ACB, Nantes, France) equipped with a temperature regulator device. The pressure transmitting fluid
128 used was distilled water. The samples were inserted into the vessel (whose internal temperature was
129 regulated to 20 °C) and processed at a compression rate of 3.4 MPa/s until the targeted pressure was
130 reached. The pressure level was held for 5 min, and decompression was nearly instantaneous (less
131 than 2 s). Water temperature reached 24 °C at 500 MPa because of the adiabatic heating. Once
132 treated, packs were then stored at 8°C and one portion was used at day 1, 5, 12, 30 for bacterial
133 enumeration and bacterial pellet collection for further DNA extraction. Unpressurized samples were
134 also included as controls.

135 **2.5. DNA preparation and amplicon sequencing**

136 Total genomic DNA was extracted from the bacterial pellet as described previously (Poirier et al.,
137 2018) using the PowerFood™ Microbial DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, USA)
138 and the High Pure PCR Template Preparation kit (Roche Diagnostics Ltd, Burgess Hill, West Sussex,
139 UK). For each sample, both DNA extracts were pooled. Then, this DNA sample was used as template
140 for three independent amplifications using either the 16S V3-V4 region of the rRNA encoding gene or
141 an internal 280 bp fragment of the Gyrase B subunit encoding gene *gyrB*, as described previously
142 (Poirier et al., 2018). All PCRs were performed in triplicate. Replicates were pooled and the amplified
143 DNA was purified with a QIAquick kit (Qiagen, Hilden, Germany). Amplicon size, quality, and quantity
144 were checked on a DNA1000 chip (Agilent Technologies, Paris, France). The MiSeq Reagent Kit v2
145 (2x250 paired-end reads, 15 Gb output) was used according to the manufacturer's instructions for
146 library preparation and sequencing on a MiSeq 2 (Illumina, San Diego, USA). The quality of the

147 obtained sequences was checked with FastQ files generated at the end of the run (MiSeq Reporter
148 Software, Illumina, USA) and additional PhiX Control. The corresponding pairs of sequences were
149 then attributed to their respective samples using the individual multiplexing barcodes. Quality
150 controls indicated a PHRED quality score of at least 30 for 94% of the reads and a median number of
151 65,520 ± 14,180 reads per sample and 95,860 ± 11,300 reads per sample for 16S rDNA and *gyrB*
152 amplicons, respectively.

153 **2.6. Operational Taxonomic Unit analysis and accession numbers.**

154 For each sample, paired-end sequences were then loaded in the FROGS (Find Rapidly OTUs with Galaxy
155 Solutions) pipeline (Escudié et al., 2018), checked for quality and assembled. We retained merged
156 sequences with a size of 280 ± 50 bp for *gyrB* and 450 ± 50 bp for 16S rRNA gene. The merged
157 (assembled) sequences have been deposited in the Sequence Read Archive database under the
158 accession numbers SAMN13761947 to SAMN13762161, corresponding to BioProject PRJNA599607.
159 SWARM clustering (Mahé et al., 2014) was applied on the assembled sequences using a maximal
160 aggregation distance of three nucleotides for 16S rRNA gene sequences; for *gyrB* sequences, clustering
161 was more stringent, with a maximal aggregation distance of two nucleotides in order to potentially
162 assign OTUs to the subspecies level. After chimeras and spurious OTUs (low-abundance and low
163 frequency OTUs arising from sequencing artifacts) removal as described previously (Poirier et al.,
164 2018), the dataset comprised 69 OTUs for the 16S rDNA dataset and 252 OTUs for the *gyrB* dataset.
165 Taxonomic assignment of 16S rDNA OTUs was performed with the Blastn+ algorithm (Camacho et al.,
166 2009) on the SILVA 128 SSU database (Quast et al., 2012), using a threshold of 97% identity for species
167 assignment. For *gyrB* OTUs, it has been previously demonstrated that PCR amplification of *gyrB* can
168 also recover the paralogous *parE* gene, which encodes the β subunit of DNA topoisomerase IV. In order
169 to determine which of the two genes had been amplified for each species, the OTU sequences were
170 blasted against the *gyrB/parE* databases established by Poirier and colleagues (Poirier et al., 2018).
171 Both *gyrB* and *parE* OTUs were retained in our diversity analysis. We assigned taxonomy to the species
172 level when 95% of a sequence matched over 90% of length coverage found in the database. The last

173 step consisted in comparing the taxonomic assignments obtained from the 16S rDNA and the *gyrB*
174 amplicon sequencing. The taxonomic assignment of the 16S rDNA-based OTUs were then
175 homogenized and improved by those obtained from the *gyrB* analysis. This strategy was particularly
176 applied to 16S rRNA gene OTUs with no clear assignment to the species-level (several species included
177 at the threshold of 97% identity).

178 2.7. **Beta-diversity and statistical analysis**.

179 Bacterial diversity analyses were performed using the phyloseq package (v1.24.2) of R (McMurdie
180 and Holmes, 2013). For each of the samples, analysis of diversity was either performed at the OTU
181 level or at the species level. In studies at the species level, OTUs with similar taxonomic assignment
182 (both *gyrB* and 16S rDNA) were merged using the TAX_GLOM function of phyloseq package, and their
183 abundance (number of reads) was averaged. This resulted into a dataset of 56 species with different
184 taxonomic assignment. Similarly, comparative analysis between ham samples with different
185 experimental conditions was performed after technical (16S rDNA/*gyrB*) and biological (biological
186 repetition) data were merged with the phyloseq function MERGE_SAMPLES. In this case, species
187 abundance was averaged between the four replicates. Bacterial diversity was compared among
188 different groups of samples with permutational ANOVA, specifically using the adonis function within
189 the vegan package (Oksanen et al., 2019).

190 **3. Results**

191 3.1. **Diced cooked ham selection for microbiota recovery.**

192 Preliminary tests were performed on diced cooked ham of different brands collected from local
193 supermarkets, as close as possible to the production date. These were then incubated under vacuum
194 or air packaging at 4°C or 8°C. We observed a large variation of initial total viable counts between
195 samples, ranging from 2 to 7 log CFU. g^{-1}. No strong influence of packaging was observed on
196 psychrophilic and mesophilic total counts, or MRS counts. Enumeration on MRS plates containing
197 bromocresol green enabled us to estimate the putative diversity of LAB species present in dices.

198 Only one to 3 types of colonies were detected indicating a poor diversity among the most dominant
199 LAB species.
200 Since our objective was to recover microbiota for further re-inoculation on cooked diced ham before
201 HPP treatment, we aimed at collecting standardized quantities of bacteria, as diverse as possible, and
202 enough DNA for further amplicon sequencing. For that purpose, we designed the sampling as
203 follows: i) diced cooked ham were sampled from supermarkets as close as possible to their
204 production date; ii) immediately after arrival in the laboratory dices were reconditioned under
205 vacuum packaging and under air to eventually increase the bacterial diversity to be collected; iii)
206 packs were then stored at 4°C and analysed every 7 days; iv) when total viable counts reached about
207 7 log CFU. g^{-1}, microbiota were collected and stored for further analyses and DNA was extracted for
208 amplicon sequencing. Through this strategy, diced cooked ham samples from four different brands
209 out of ten (further referred as HAM_A to HAM_D samples) were obtained with enough bacterial
210 diversity and DNA quality (Table 1). No significant difference in population level was observed
211 between air and vacuum packaging, except in HAM_B for which both PCA and MRS counts were
212 about 1 log higher (respectively 7.97 *vs* 6.53 log CFU g^{-1} and 7.74 *vs* 6.82 log CFU g^{-1}) after storage
213 under air.

214 **3.2. The four types (brands) of diced cooked ham can be distinguished by the diversity of their**
215 **bacterial communities.**

216 The bacterial diversity was estimated using a combination of two amplicon sequencing strategies,
217 one using V3-V4 region of the 16S rRNA gene and another one using an internal ~280 bp fragment of
218 the gyrase B subunit encoding gene (*gyrB*) in order to improve taxonomic assignment of the OTUs to
219 the species or sub-species level as described previously (Poirier et al., 2018). These two strategies
220 were also used as technical replicates for ensuring that no biases were obtained in the
221 characterization of the four different microbiota as observed in Figure 1A. Only slight differences
222 between both strategies were observed in the clustering of air samples of HAM_C and D. The
223 average number of species detected per sample was 38 ± 5. As shown in Figure 1B, HAM_C and

224 HAM_D samples showed a highly similar microbiota dominated by Firmicutes, in particular
225 *Latilactobacillus sakei* and *Leuconostoc carnosum*, two species commonly found on this type of cured
226 meat products (Raimondi et al., 2019; Vasilopoulos et al., 2010; Zagdoun et al., 2019). Furthermore,
227 storage type under vacuum or air packaging did not influence significantly the overall abundance of
228 the identified species. On the other hand, the bacterial communities characterized from samples of
229 HAM_A and HAM_B were significantly different from each other and from the aforementioned
230 products, in particular with a clear dominance of Proteobacteria over Firmicutes (Figure 1C). Ham_A
231 microbiota was mainly composed of *Pseudomonadaceae* and *Enterobacteriaceae* with *Pseudomonas*
232 *lundensis* and *Serratia grimesii* being the most abundant species for Proteobacteria and
233 *Carnobacterium maltaromaticum* being the most abundant species for Firmicutes. Samples from
234 Ham_B revealed to contain the most original microbiota among the four types of ham with the three
235 most abundant 16S rDNA or *gyrB* OTUs being assigned to unknown species as the identity
236 percentage of these OTUs to *gyrB* gene in databases was lower to the ANI (Average Nucleotide Index)
237 threshold of 95% classically used for bacterial species boundary (Qin et al., 2014). The most
238 abundant Firmicutes *gyrB* OTU was assigned to a *Carnobacterium sp.* showing only 89.0% identity (as
239 best match) to *gyrB* gene of *Carnobacterium funditum* strain DSM 5970. Among Proteobacteria, one
240 OTU was assigned to a *Psychrobacter sp.* with closest match was 89.9% to *gyrB* gene of
241 *Psychrobacter cryohalolentis* FDAARGOS_308, a strain isolated from Siberian permafrost (GenBank:
242 GCF_002208775.2). For the second Proteobacteria OTU, the best identity score found was 91.6 %
243 with *Vibrio sp. SM1977 gyrB* gene, a species isolated from coralline algae surface (GenBank:
244 CP045699.1). It should be noted that the abundance of these two uncharacterized species
245 (*Psychrobacter sp.* and *Vibrio sp.*) varied significantly according to the packaging type used for
246 storage, which may correlate with their 10 times higher counts on air stored samples (Table 1),
247 although we have no evidence for their cultivability in our growth conditions.
248
249 **3.3. Combined effect of HPP and biopreservation on bacterial community dynamics.**

250 For the further steps of our analysis dedicated to monitoring the effect of HPP and biopreservation
251 by *L. lactis* CH-HP15 on the ham microbiota dynamics during storage at 8°C, we decided to focus on
252 the two different microbiota from HAM_A and HAM_B. This was based on the fact that these two
253 microbiota showed a community structure enriched in bacterial species with highly spoilage
254 potential (Proteobacteria) in comparison to microbiotas of HAM_C and HAM_D enriched in
255 *Latilactobacillus*; those latter species being generally rather considered as positive microbial
256 component (Vermeiren et al., 2006, 2004). In addition, we chose HAM_B microbiota because of the
257 unexpected presence of the two uncharacterized *Psychrobacter* and *Vibrio* species. The experimental
258 strategy was based on re-inoculation at the level of 4 log CFU. g^{-1} of the original microbiota from both
259 HAM_A and HAM_B on minimally contaminated diced cooked ham with reduced nitrite content (25
260 mg/kg). For both types of microbiota, an equimolar amount mixture of microbiota from air and
261 vacuum storage previously stored at -80°C was used. The *L. lactis* CH-HP15 bioprotective strain was
262 inoculated at a higher level of 7 log CFU. g^{-1} to enable regrowth and ensure efficient biopreservation
263 activity taking into account a possible high inactivation following the HPP treatment (Ramaroson et
264 al., 2018). Table 2 is summarising the estimated bacterial population level at different storage times
265 after the several processing treatments. Similarly, Figure 2 is depicting the relative abundance of
266 each species via 16S rDNA and *gyrB* amplicon sequencing.
267
268 The control samples without any processing treatment revealed that at day 1 after inoculation, the
269 microbial communities were similar to the microbiota from HAM_A and HAM_B analyzed in Figure 1,
270 showing that the dices prepared for the challenge-tests were indeed poorly contaminated and that
271 the microbiota had recovered with no strong bias from freezing. HAM_A microbiota grew rapidly
272 under vacuum from ~4 log CFU. g^{-1} to reach ~7 log CFU. g^{-1} at day 5 and ~8 log CFU. g^{-1} at day 30. In
273 contrast, HAM_B microbiota showed a more reduced growth dynamics, reaching at most ~7 log CFU.
274 g^{-1} during the whole storage period. Interestingly, the structure of the communities also changed
275 during storage: in HAM_A samples, *P. lundensis*, a species abundant at the beginning of storage was

276 progressively overgrown by *S. grimesii* whereas, *C. maltaromaticum,* the third abundant species,
277 remained at a stable proportion within the community; in HAM_B, the dominant *Vibrio sp.* was also
278 overgrown over time by *Lactococcus piscium*, although this latter species was largely subdominant
279 (<0.1%) in the initial microbiota.
280
281 Processing with biopreservation by *L. lactis* CH-HP15 led to two different effects regarding samples
282 from HAM_A and HAM_B. In HAM_A samples, the addition of *L. lactis* had a strong reduction effect
283 on the abundance of Firmicutes species, in particular on *C. maltaromaticum*, which thus became
284 largely subdominant, whereas *B. thermosphacta* was temporally (up to 12 days of storage) and
285 presumably taking benefit from this change (figure 2A). Meanwhile, the inoculation of *L. lactis* had no
286 obvious effect on Proteobacteria species whose pattern was unchanged overtime compared to
287 control. Indeed, after 30 days of storage, while the microbiota had reached a population level above
288 8.5 log CFU. g^{-1}, *P. lundensis* and *S. grimesii* species displayed a relative abundance of about 50% of
289 the microbiota; the remaining part of the microbiota being covered by *L. lactis,* although this strain
290 had been inoculated thousand-fold higher than the original microbiota. In HAM_B, the growth
291 dynamics of the dominant *Vibrio sp.* was also not affected in comparison to the untreated samples.
292 However, the addition of *L. lactis* induced a possible competition with *L. piscium*, resulting over time
293 into equilibrium of both species. More generally, *L. lactis* CH-HP15 was less competitive in microbiota
294 from HAM_B samples than in microbiota from HAM_A samples.
295 Whether or not biopreservation was applied, HPP treatment resulted in major changes in the growth
296 dynamics of both microbiota. HPP treatment alone on HAM_A samples reduced drastically the
297 viability of the original microbiota which remained below 4 log CFU. g^{-1} after 30 days of storage
298 (Table 2). It is likely that bacterial cells were severely injured, indeed no DNA could be recovered
299 from the bacterial pellet despite the use of several extraction protocol trials. Therefore, diversity
300 analysis by amplicon sequencing could not be performed. Nevertheless, samples treated with both

301 HPP and inoculation by *L. lactis* enabled better recovery of DNA and could provide an overview of the
302 HPP treatment on the HAM_A original microbiota.
303 Proteobacteria species were the most impacted by high pressure treatment, whereas *L. Lactis* CH-
304 HP15 could recover rapidly from this treatment reaching progressively a population level above 7.5
305 log CFU. g^{-1} after 30 days, and almost a complete domination of the microbiota. Interestingly, as
306 described above on samples only treated with *L. lactis*, the addition of the bioprotective
307 microorganism increased temporally (up to 12 days of storage) the relative proportion of *B.*
308 *thermosphacta*, thereby indicating that this latter species is quite resistant to HPP treatment. Unlike
309 microbiota from HAM_A samples, that from HAM_B samples recovered rapidly from HPP treatment
310 alone, almost reaching the population level of untreated samples at 30 days (6 log CFU.g in average).
311 The community structure was itself barely affected by the HPP treatment with only a higher
312 proportion of *Psychrobacter sp.* at longer storage time in comparison to untreated samples.
313 Processing with both HPP and *L. lactis* inoculation confirmed these observations as the population
314 level, combining both initial microbiota and *L. lactis* , recovered rapidly (12 days) to 7.0 log CFU. g^{-1}
315 and raised further to ~10 log CFU. g^{-1} at 30 days, which is almost 4 log higher than the level in
316 untreated ham. However, the HPP treatment was more favorable to Firmicutes species in the end,
317 leading to a domination of *L. lactis* CH-HP15 together with *B. thermosphacta.*
318

319 **4. Discussion**

320 The use of an appropriate HPP treatment to preserve perishable food has been the focus of many
321 studies and reviews (Oliveira et al., 2015; Yamamoto, 2017). HPP is being used to enhance food
322 safety by reducing the microbial development in final products, meanwhile maintaining at best their
323 nutritional and sensorial values to a level acceptable to consumers (Pietrasik et al., 2016). However,
324 the effect of HPP on microbial growth dynamic and community structures has not been widely
325 studied and it is not known whether hurdle strategies should be applied in combination with HPP to
326 efficient microbiota inactivation or stabilisation. Indeed, a recent work carried out with a simplified

327 ham microbiota (five species including *Listeria monocytogenes, L. sakei, B. thermosphacta, C.*
328 *maltaromaticum* and *Leuconostoc gelidum*) showed that HPP treatment is not sufficient to inhibit
329 growth recovery of the microbiota over long storage time (Teixeira et al., 2018). This is pointing out
330 that there is a clear gap in our knowledge on the HPP efficiency towards various microbial
331 communities which may be present on cooked ham. Although the technology could be a very
332 promising strategy to improve the safety of nitrite-reduced cooked ham, it is necessary to investigate
333 whether it should be used as hurdle with the combination of other strategies such as biopreservation
334 (Ramaroson et al., 2018).
335 In this context, our study provides data in link with the following questions: does HPP display a
336 generic effect on microbial inactivation or is this inactivation a microbiota-dependent phenomenon?
337 Is the use of nisin-producing *L. lactis* mediated biopreservation a worth hurdle strategy when
338 combined with HPP, and, is this biopreservation effect also microbiota dependent?
339
340 Our first strategy was to recover microbiota from several types of cooked ham (different brands)
341 stored under air or vacuum. As previously demonstrated by Raimondi and colleagues (Raimondi et
342 al., 2019), the microbiota of the four types of cooked ham was poorly diverse but highly variable.
343 Some cooked hams with poor diversity were dominated by *L. sakei, Latilactobacillus curvatus* and *L.*
344 *carnosum* and their microbiota were not considered further as HPP treatments were previously
345 demonstrated to be efficient against the growth of these species (Han et al., 2011). On the other
346 hand, cooked hams from two other producers were characterized by a more diverse microbiota.
347 These microbiota were composed of a quite different mixture of species from Firmicutes and
348 Proteobacteria phyla, including not yet characterized dominant species. Therefore, these two types
349 of microbiota were chosen as model microbiota to test the efficiency of HPP and biopreservation.
350
351 Firstly, our results demonstrate that the reduction of viable cells population by HPP is slightly more
352 effective on Proteobacteria species than on Firmicutes species resulting in a small shift of the

353 dominant population from Proteobacteria to Firmicutes when HPP is applied. This finding
354 corroborates many previous observations made by comparing individual species of Gram-negative
355 and Gram-positive pathogenic bacteria resistance to HPP for instance (see Oliveira et al., 2015 for a
356 review). Although the cell surface morphology of both types of bacteria can explain such difference,
357 it is interesting to notice that the recovery of cells after HPP is species (and *per se* microbiota)
358 dependent. Although both microbiota from HAM_A and HAM_B were re-inoculated at the same
359 population level before HPP treatment, that from HAM_A, composed of *P. lundensis* and *S. grimesii*
360 revealed more sensitive (no or almost no recovery) to HPP than that from HAM_B composed of
361 uncharacterised *Psychrobacter* and *Vibrio* species. The species or even strain-dependent resistance
362 to HPP has already been pointed out for Firmicutes, in particular for *L. monocytogenes* and
363 *Staphylococcus aureus* (Jofré et al., 2008b). The underlying mechanisms are not fully understood as
364 HPP resistance and recovery of bacteria also depend on the cell physiology of bacteria present on the
365 food matrix before the treatment. As well, the matrix composition may influence bacterial recovery
366 (Tassou et al., 2007), as was shown for fat content influencing *L. monocytogenes* recovery from
367 thermal inactivation (Verheyen et al., 2020). Furthermore, the capacity of the bacteria to resist HPP
368 treatment should be dissociated from the ability of the bacterial cells to recover and thrive during
369 the storage conditions. Our data are a good illustration of this phenomenon. For instance, *S. grimesii*
370 revealed more competitive than *P. lundensis* to grow during 30 days at low temperature and perhaps
371 under vacuum packaging, leading to a switch of the two species during storage. Similarly, *L. piscium*,
372 a sub-dominant species in original HAM_B microbiota, outcompeted the initial dominant *Vibrio*
373 species.
374 Another finding from our work is that the *L. lactis* strain used for biopreservation is not competitive
375 towards ham original microbiota. Although, we noticed that it has perceivable effect towards the
376 reduction of other Firmicutes species (*e.g*. *C. maltaromaticum*), perhaps due to the production of
377 nisin. Indeed, this bacterium has been shown in previous studies to harbour sensitivity to this
378 bacteriocin in vacuum packed meat (La Storia et al., 2012; Teixeira et al., 2018). Albeit the *L. lactis*

379 CH-HP15 strain was inoculated with a level three orders of magnitude higher than the original ham
380 microbiota, the strain was found between 25% to 50% of overall relative abundance in HAM_A and
381 HAM_B, respectively. We previously observed that the inactivation level of this strain after HPP was
382 more important than that of other LAB species, which was compensated by its better ability to
383 recover and rapidly regrow after the treatment (Ramaroson et al., 2018). The lack of *L. lactis*
384 competitiveness is probably due to the specific ecology of ham. Indeed, *L. lactis* CH-HP15 was shown
385 able to grow in sterile diced cooked ham at 8°C, reaching 9 log CFU g^{-1} within 5 days with an initial
386 inoculum of 6 log CFU g^{-1} (Ramaroson et al., 2018). In the present study, the presence of species
387 belonging to the natural microbiota and thus potentially better adapted may explain the lack of
388 fitness of *L. lactis* CH-HP15. Nevertheless, the combination of high level of inoculation of *L. lactis* with
389 HPP treatment leads to an efficient stabilisation of the original cooked HAM_A microbiota, by limiting
390 the growth of *P. lundensis* and *S. grimesii*. Unlike this situation, results were different for HAM_B
391 samples for which the hurdle strategy could not trigger, over the storage time, the outcompetition of
392 *L. lactis* versus the original HAM_B microbiota. Therefore, it can be concluded that for microbiota of
393 HAM_B, the hurdle strategy failed in stabilizing and inactivating the microbial growth.
394 Furthermore, our results show that *B. thermosphacta* is a species with a very strong recovery
395 dynamics following the HPP treatment. Such observation has already been made by Teixiera and
396 colleagues using the simplified ham microbiota described above (Teixeira et al., 2018). This finding
397 raises the question of HPP treatment benefit on *B. thermosphacta* containing microbiota as this
398 species is a well-known meat spoilage micro-organism (Illikoud et al., 2019).
399

400 **5. Conclusion**

401 From our work, we thus conclude that both HPP treatment and *L. lactis*-based biopreservation is
402 strongly microbiota dependent and thus, the value of this strategy requires a specific assessment for
403 each type of cooked ham production. We recommend that HPP treatment should be evaluated not

404 only for pathogenic bacteria but also on putative spoilage bacteria in order to estimate the specific

405 selection of these undesirable micro-organisms, in particular *B. thermosphacta.*

406

407 **Legends to the figures.**

408 **Figure 1. Comparative bacterial community composition between the four brand of cooked ham**

409 **analysed.**

410 **Panel A:** Cooked ham samples unsupervised clustering tree based on Bray-Curtis distance and Ward

411 algorithm. Samples are colored according to the cooked ham brand. Both air (AIR) and vacuum

412 packed (VPA) samples as well as 16 S rDNA-based or *gyrB* –based analysis are shown. **Panel B and C**

413 show a bar plot composition of the top 10 species identified among Firmicutes and Proteobacteria

414 phyla, respectively. Species are plotted according to their relative abundance in percentage of the

415 whole microbiota. Novel genus nomenclature was applied for *Latilactobacillus* species (*L. sakei* and *L.*

416 *curvatus*) as recently proposed by Zheng and colleagues (Zheng et al., 2020).

417 **Figure 2. Time course dynamic barplot composition of bacterial communities in nitrite-reduced**

418 **cooked ham samples upon various treatments.**

419 Data for HAM_A and HAM_B samples are shown in panel A and B, respectively. Species are plotted

420 according to their relative abundance in percentage of the whole microbiota. The *L. lactis* strain CH-

421 HP15 used for biopreservation is highlighted in green in the left legend. Each bar plot is the mean of

422 two biological replicates and each replicate value being the average of both 16S rDNA and *gyrB*

423 amplicon sequencing data. As described in Figure 1 for *Latilactobacillus* species, novel genus

424 nomenclature was applied for *Dellaglioa algida* (former *Lactobacillus algidus*) as recently proposed

425 by Zheng and colleagues (Zheng et al., 2020).

426

427 **Conflict of interest**

428 None. The funders had no role in the study's design, data collection, and interpretation, or the

429 decision to submit the work for publication.

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Table 1 Description of ham specificities

Sample name according to brands	Sampling date	Additives[a]	Total counts[b] log CFU g^{-1}	LAB counts[c] log CFU g^{-1}
HAM_A	UBD + 6 days Dec 2 2015	Dextrose Sodium nitrite E250 Potassium chloride E508 Sodium erythorbate E316 Potassium lactate E326 Sodium ascorbate E301 Sodium acetate E262 Diphosphates E450 Triphosphates E451 Polyphosphates E452	8.6 ± 0.4	8.2 ± 0.0
HAM_B[d]	UBD + 5 days Mai 18 2016	Sugar Lactose Nitrite salt Common salt Spices	7.2 ± 0.7	7.3 ± 0.5
HAM_C	UBD – 1 day Nov 6 2015	Dextrose Sodium nitrite E250 Potassium chloride E508 Potassium lactate E326 Sodium acetate E262 Sodium ascorbate E316 Polyphosphates E452	8.6 ± 0.1	9.0 ± 0.1
HAM_D	UBD – 4 days Aug 21 2015	Sodium nitrite E250 Potassium chloride E508 Potassium lactate E326 Sodium diacetate E262 Sodium ascorbate E316	10.3 ± 0.1	8.1 ± 0.1

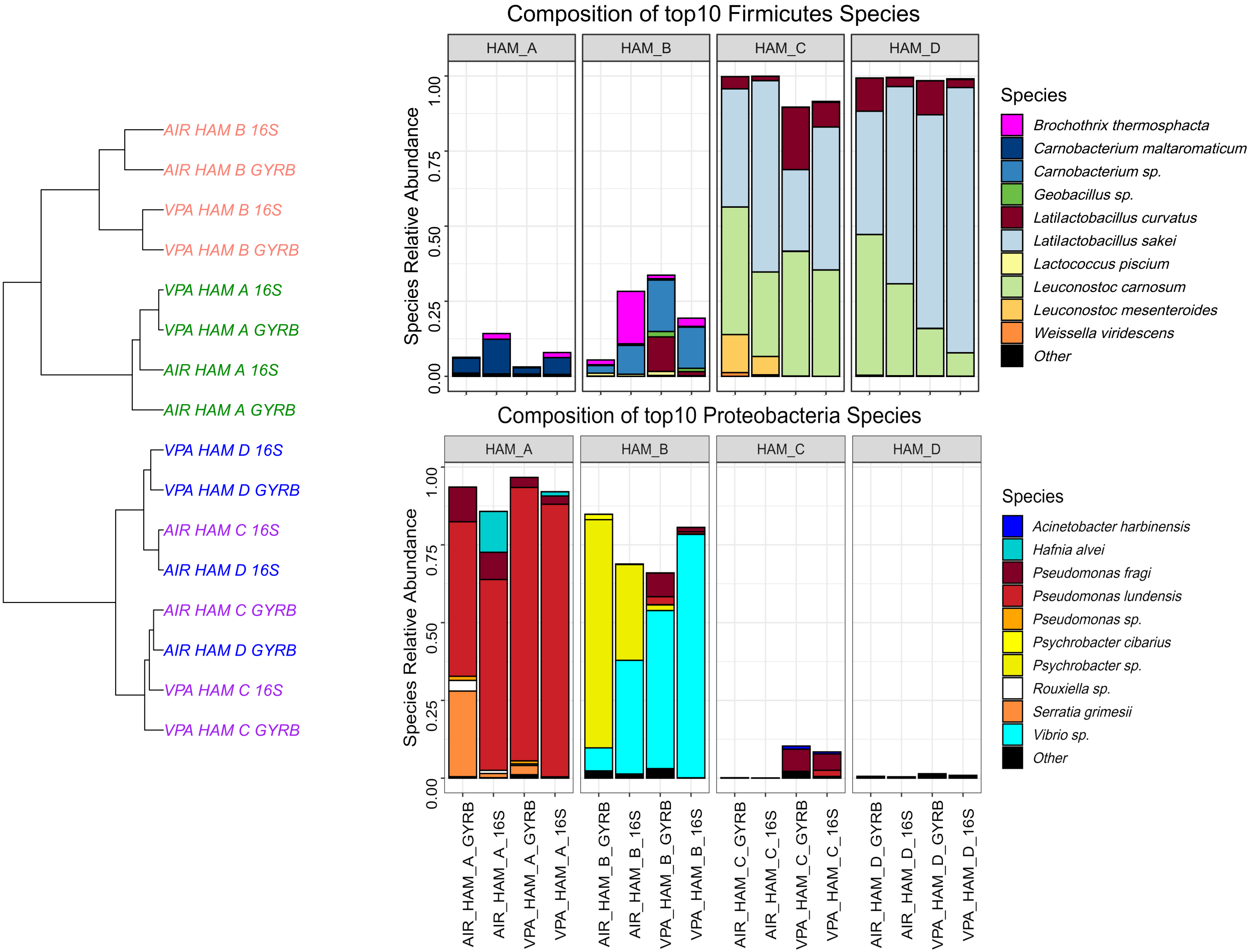
[a]: from information provided on the packaging. [b]: PCA count mean values from portions stored under vacuum and air packaging . [c]: MRS count mean values from portions stored under vacuum and air packaging. [d]: brand sold as organic.

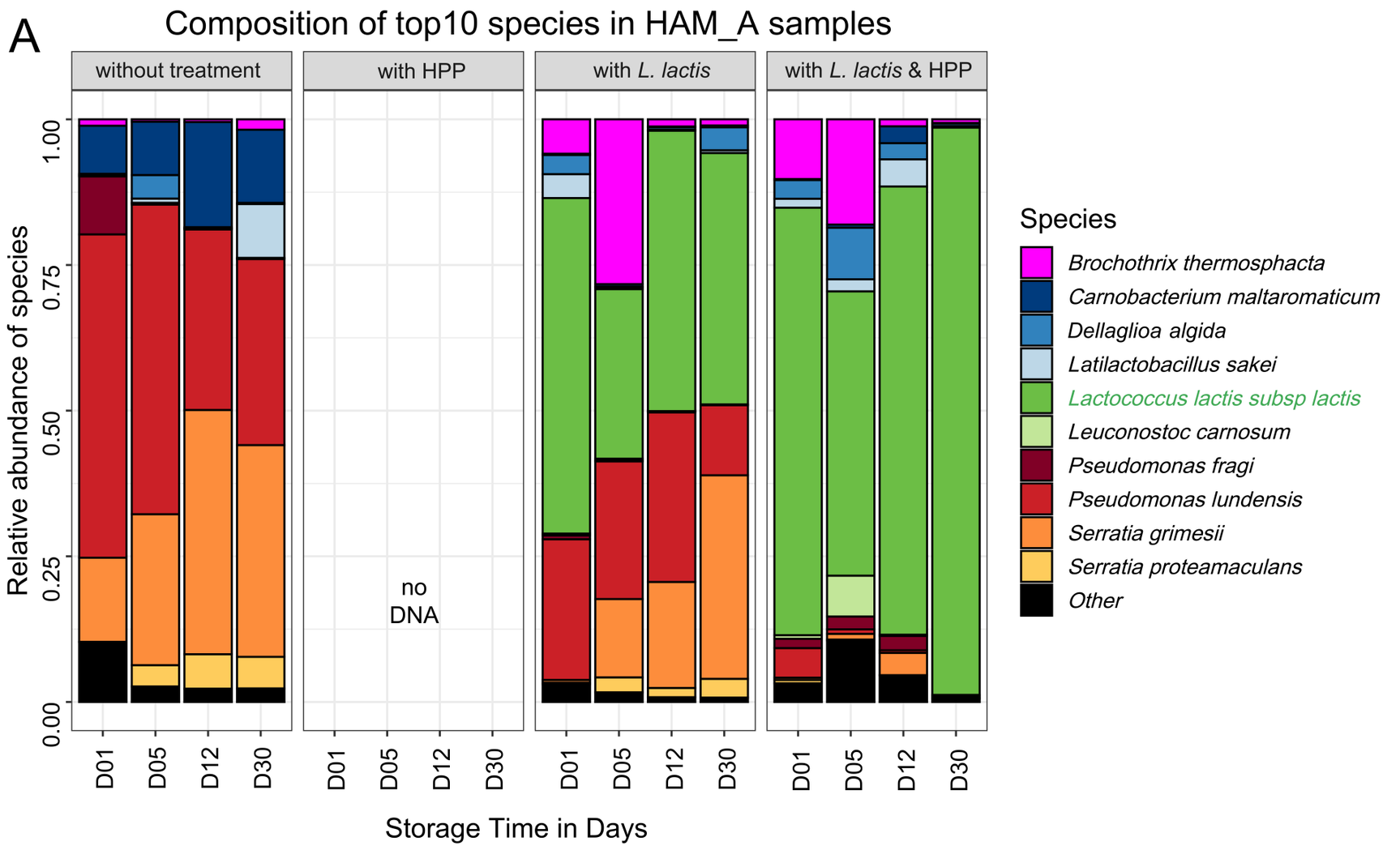
Table 2.

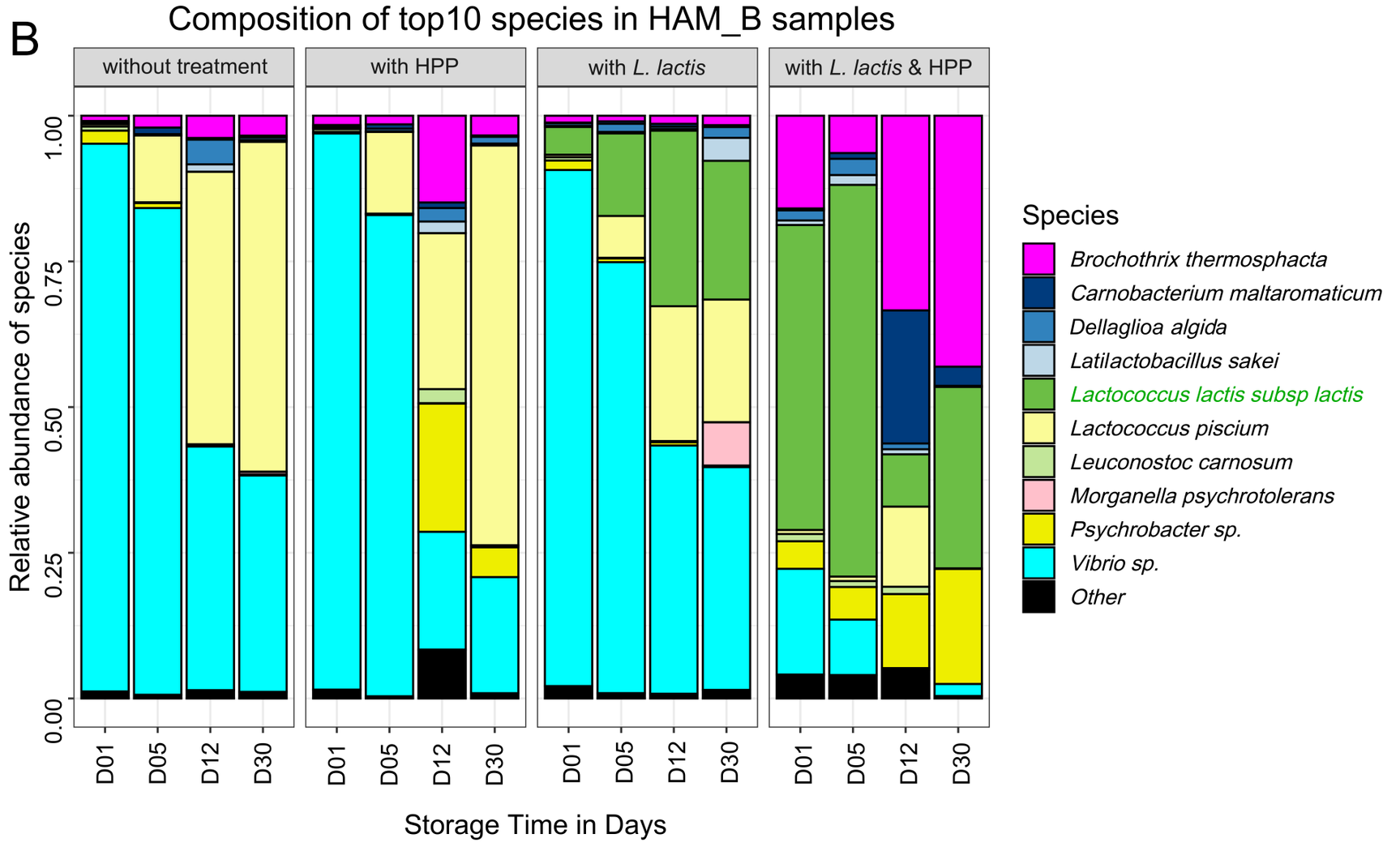
	Population level in log CFU.g^{-1} of nitrite-reduced diced cooked ham			
	Storage time in days[A]			
HAM_A samples	D01	D05	D12	D30
Without treatment	4.63 ± 0.09	7.32 ± 0.14	7.84 ± 0.10	8.15 ± 0.15
With HPP	1.20 ± 0.14	3.74 ± 0.01	3.00 ± 1.00	4.02 ± 0.59
With L. lactis	7.21 ± 0.62	9.18 ± 0.02	8.39 ± 0.12	8.87 ± 0.87
With L. lactis and HPP	2.54 ± 0.45	3.97 ± 0.26	5.42 ± 1.27	7.60 ± 0.03
HAM_B samples	D01	D05	D12	D30
Without treatment	5.24 ± 0.18	6.10 ± 0.13	6.90 ± 0.33	6.50 ± 0.20
With HPP	1.39 ± 0.09	ND[B]	5.33 ± 0.34	6.23 ± 0.42
With L. lactis	7.25 ± 0.51	8.29 ± 0.01	7.82 ± 0.21	8.50 ± 0.50
With L. lactis and HPP	2.75 ± 0.44	3.64 ± 0.07	7.11 ± 0.13	10.2 ± 0.20

[A] Population data were calculated and averaged from the two biological replicates and from two plating culture condition enumerating both total mesophilic bacteria and lactic acid bacteria (see M&M).

[B] Not Determined.


AIR HAM B 16S
AIR HAM B GYRB
VPA HAM B 16S
VPA HAM B GYRB
VPA HAM A 16S
VPA HAM A GYRB
AIR HAM A 16S
AIR HAM A GYRB
VPA HAM D 16S
VPA HAM D GYRB
AIR HAM C 16S
AIR HAM D 16S
AIR HAM C GYRB
AIR HAM D GYRB
VPA HAM C 16S
VPA HAM C GYRB
Composition of top10 Firmicutes Species
HAM_A
HAM_B
HAM_C
HAM_D
Species Relative Abundance
0.00
0.25
0.50
0.75
1.00
Species
Brochothrix thermosphacta
Carnobacterium maltaromaticum
Carnobacterium sp.
Geobacillus sp.
Latilactobacillus curvatus
Latilactobacillus sakei
Lactococcus piscium
Leuconostoc carnosum
Leuconostoc mesenteroides
Weissella viridescens
Other
Composition of top10 Proteobacteria Species
HAM_A
HAM_B
HAM_C
HAM_D
Species Relative Abundance
0.00
0.25
0.50
0.75
1.00
Species
Acinetobacter harbinensis
Hafnia alvei
Pseudomonas fragi
Pseudomonas lundensis
Pseudomonas sp.
Psychrobacter cibarius
Psychrobacter sp.
Rouxiella sp.
Serratia grimesii
Vibrio sp.
Other
AIR_HAM_A_GYRB
AIR_HAM_A_16S
VPA_HAM_A_GYRB
VPA_HAM_A_16S
AIR_HAM_B_GYRB
AIR_HAM_B_16S
VPA_HAM_B_GYRB
VPA_HAM_B_16S
AIR_HAM_C_GYRB
AIR_HAM_C_16S
VPA_HAM_C_GYRB
VPA_HAM_C_16S
AIR_HAM_D_GYRB
AIR_HAM_D_16S
VPA_HAM_D_GYRB
VPA_HAM_D_16S


A
Composition of top10 species in HAM_A samples
without treatment
with HPP
with L. lactis
with L. lactis & HPP
Relative abundance of species
0.00
0.25
0.50
0.75
1.00
no
DNA
D01
D05
D12
D30
Storage Time in Days
Species
Brochothrix thermosphacta
Carnobacterium maltaromaticum
Dellaglioa algida
Latilactobacillus sakei
Lactococcus lactis subsp lactis
Leuconostoc carnosum
Pseudomonas fragi
Pseudomonas lundensis
Serratia grimesii
Serratia proteamaculans
Other


B
Composition of top10 species in HAM_B samples
without treatment
with HPP
with L. lactis
with L. lactis & HPP
Relative abundance of species
0.00
0.25
0.50
0.75
1.00
D01
D05
D12
D30
Storage Time in Days
Species
Brochothrix thermosphacta
Carnobacterium maltaromaticum
Dellaglioa algida
Latilactobacillus sakei
Lactococcus lactis subsp lactis
Lactococcus piscium
Leuconostoc carnosum
Morganella psychrotolerans
Psychrobacter sp.
Vibrio sp.
Other